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<p>This report describes the development and synthesis and assaying of a new class of Yop51 inactivators. The conclusions from this work are:</p> <ul style="list-style-type: none"> (1) α-bromoaryphosphonate is the basic inactivator molecule active against Yop51; (2) planar aromatic systems appear to bind well to the enzyme active site; (3) incorporating the basic inactivator functionality into a benzodiazepine template yielded the most potent inactivator. 				
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FOREWORD

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The Role of Phosphotyrosine Phosphatases in Breast Cancer

Introduction

The formation and cleavage of phosphate ester bonds are common biochemical events involved in many biological processes such as signal transduction, energy storage, and protein phosphorylation. The phosphorylation state of protein in cells is controlled by two enzyme families, protein kinases and phosphatases. The involvement of a number of protein tyrosine kinases and protein tyrosine phosphatases (PTPases) in regulating signal transduction has recently been identified.¹ Signal transduction is the mechanism by which external signaling stimuli, such as hormones and cytokines, transmit information across the cell membrane. This information modulates cellular processes such as metabolism, transcription and translation of DNA, cell division, cell growth, cellular transformation, and apoptosis.²

Many tyrosine kinases have been shown to be oncogenic. Abnormal tyrosine kinase activity is often associated with cell transformation. On the other hand the role of PTPases in cell transformation is still unclear. It was formerly accepted that PTPases functioned merely to reverse the activity of tyrosine kinases.³ In the light of the discovery of transmembrane PTPases, the relationship between kinases and phosphatases is believed to be more complex than the simple antagonists. Moreover, the overexpression of PTP α (a soluble PTPase) in rat embryo fibroblasts causes the activation of *pp60src* by dephosphorylation of the negative regulatory site, leading to oncogenic cell transformation.⁴ An increased level of PTPases activity was also observed in breast cancer and head and neck squamous cell carcinoma.⁵ The exact role of PTPases in these transformed cells is still unclear, although it is possible that they may play a crucial role in oncogenesis. Early efforts in the study of tyrosine phosphorylation focused on tyrosine kinases. However, with recent discoveries of different PTPases, an awareness of the importance of this enzyme family has grown considerably.

Almost all known PTPases hydrolyze *p*-nitrophenylphosphate, although the preferred substrates are phosphopeptides. Almost all known PTPases share substantial active-site homology. This, however, does not imply that all existing PTPases are homologous or use the same catalytic motif. The technique by which most known PTPases have been isolated requires such that these proteins share homology with the catalytic domain of PTP1B,⁶ the first high molecular weight PTPase isolated from human placenta. It is possible that PTPases that do not share the same catalytic domain as PTP1B have yet to be discovered. In addition, substantial sequence homology does not guarantee identical reaction pathways. Studying a number of PTPases with inactivators developed in this laboratory may help to reveal subtle differences between these enzymes.

A number of enzymological studies on PTPases, especially *yersinia* PTPase (Yop 51)⁷ and CD45 (a transmembrane glycoprotein found in hematopoietical cells)⁸ suggest that the hydrolysis of phosphatemonoesters proceeds through a phosphoenzyme intermediate. A rapid quench experiment using ³²P-labeled synthetic phosphopeptide as substrate revealed that rapid phosphorylation of CD45 occurs, followed by a slower hydrolysis process.⁹ This enzyme family share homologous region of approximately 250 amino acid long, consisting of a number of highly conserved residues.¹⁰ One of these residue is a cysteine which is believed to be the active-site nucleophile involved in phosphate hydrolysis.

A number of small compounds, such as diethyl pyrocarbonate, phenylglyoxal, iodoacetic acid, and arsenic acid, are non-specific inhibitors for PTPases. Since the preferred substrates for these enzymes are phosphopeptides, it is unclear whether these inactivating agents interact with active-site amino acid residues or with any reactive residues

in the protein. Therefore, specific PTPase inactivators are necessary tools to study this enzyme family. These inactivators can be used to study substrate specificity and the nature of active site residues. This information is valuable in designing more specific and potent inactivators which could be used in *in vitro* and *in vivo* studies of PTPases.

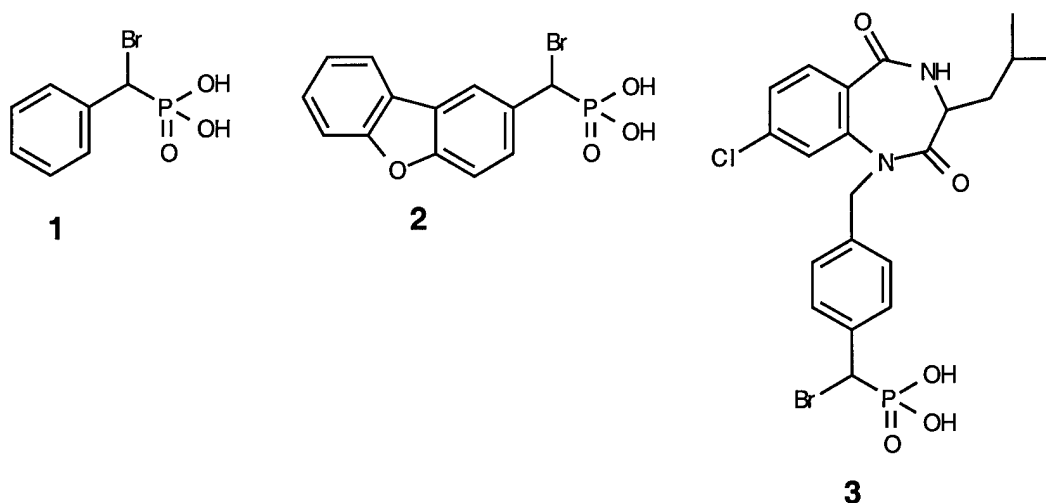
The focus of this work is to develop active-site specific and potent affinity inactivators for Yop51. This particular PTPase was selected as a representative of the enzyme family because many studies have been done on this enzyme.¹¹ These affinity reagents can potentially be used in tagging and isolating previously unknown PTPases. They may also be useful in delineating the role of PTPases in cellular signal transduction processes and their roles in tumorigenesis.

Body

Our work in developing potent affinity reagents for PTPases has resulted in three significant observations.

1. α -Bromobenzylphosphonate (compound **1**) is the basic inactivator motif identified to inactivate Yop51 in low millimolar range.
2. The α -bromophosphonate analog of dibenzofuran (compound **2**) is active against Yop51 in low micromolar range.
3. Incorporation of α -bromobenzylphosphonate into a benzodiazepine template (via a combinatorial chemistry technique) resulted in an inactivator that is approximately a hundred fold more potent than compound **2**.

Figure 1. Affinity reagents for PTPases



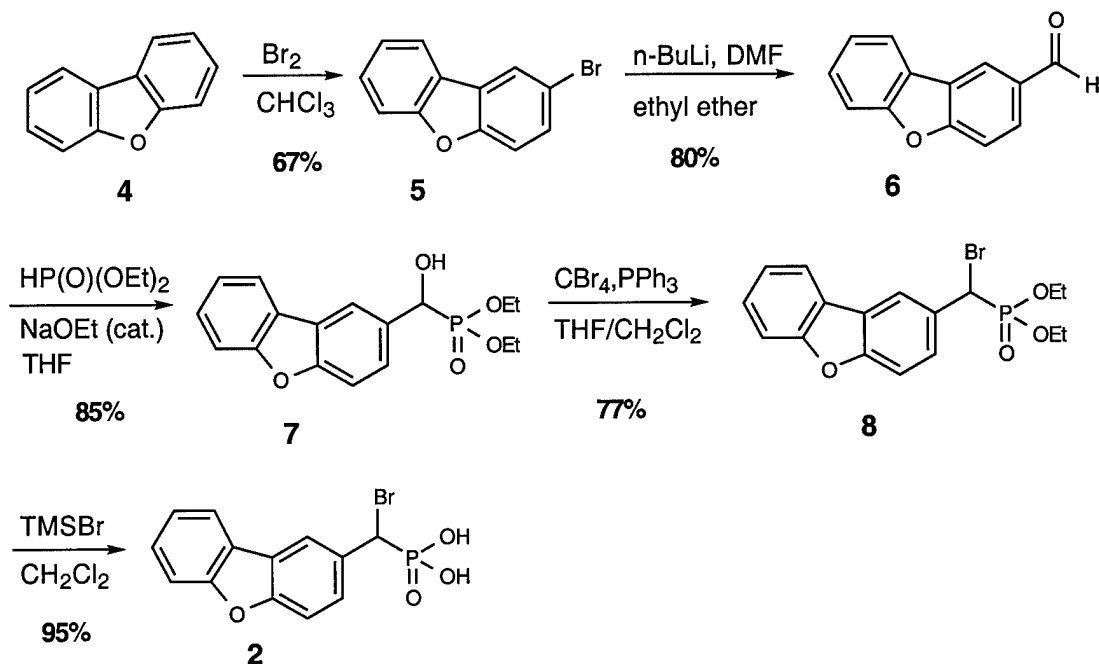
α -Bromobenzylphosphonate (compound 1).

It has been established in our laboratory that compound **1** is an affinity reagent for Yop51.¹² It is active against the enzyme in low millimolar range. This molecule inactivates by acting specifically at the enzyme active site.

α-Bromobenzyl Analog of Dibenzofuranyl (compound 2).

However, compound 1 also demonstrated some activity towards low molecular weight dual PTPase.¹³ In the efforts to develop more potent and more specific affinity reagents for Yop51, the basic motif or *α*-bromoarylphosphonate was modified by appending an electron-withdrawing or an electron-donating group at the *para* position or by extending the aromaticity. The preparation of compound 2 is illustrated in Scheme 1.

Scheme 1. Synthetic pathway for the preparation of compound 2



In addition to compound 2, eleven other compounds were prepared. These molecules were subsequently tested against Yop51. Their IC_{50} values (the concentrations required to inactivate the enzyme in 5 minutes to give 50% remaining enzymic activity) were then compared to evaluate the potency (Appendix 1).

Compound 2 has an IC_{50} value of 40 μM . It is the best of the twelve compounds synthesized and tested. The extension of aromaticity in compound 2, providing a more rigid and planar system, appears to improve binding affinity to the enzyme active site. This increased binding, in turn, may have increased the ability of the molecule to trap an active-site nucleophilic residue, leading to enzyme inactivation. An inactivation experiment was also carried out in the presence of β -naphthylphosphate, a good substrate for Yop51. In the presence of 2 mM β -naphthylphosphate, inactivation was decreased by approximately 40%. This observation revealed that compound 2 most likely inactivates specifically in the enzyme active site. Compound 2 was also tested against other family of phosphatases such as prostatic acid phosphatase, purple acid phosphatase and low molecular dual specificity phosphatase. The results indicated that it is not active against those phosphatases.

Benzodiazepine Library.

The result of the study on compound 2 prompted us to incorporate the *α*-bromoarylphosphonate motif into a planar scaffold such as a benzodiazepine molecule. Benzodiazepines are an important class of compounds commonly used as templates in

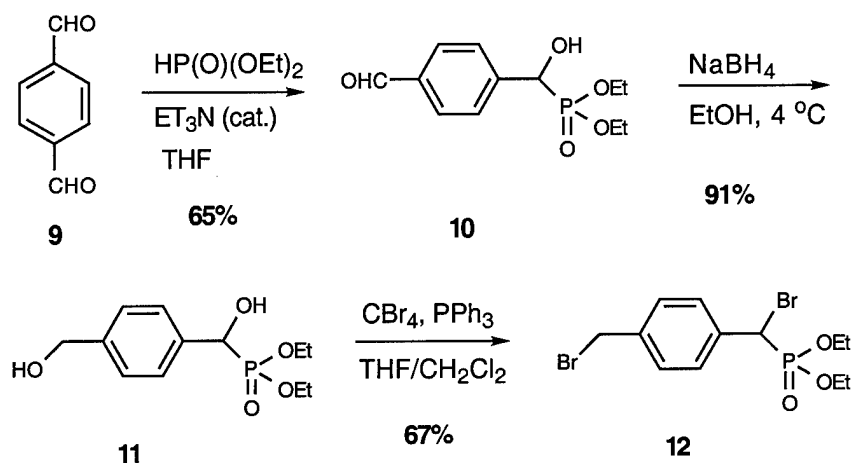
combinatorial chemistry libraries. They are good pharmacophores and have been demonstrated to have promising activities as antithrombotics.¹⁴

General methods for the solid-phase synthesis of 1,4-benzodiazepin-2,5-dione libraries have been reported in the literature.¹⁵ We chose to use the method developed by Ellman and coworkers¹⁴ because this synthetic method is mild and tolerated by an α -bromoarylphosphonate functionality.

Compound **12** was synthesized (Scheme 2) and subsequently used to prepare compound **3** (Scheme 3). In preliminary experiments, we synthesized compound **3** via both solution chemistry (Scheme 3) and solid phase chemistry. Compound **3** is a prototype inactivator having a benzodiazepin template. Initial kinetic experiments demonstrated that compound **3** is a potent inactivator for Yop51 (Appendix 2). Its approximate IC₅₀ value is between 1 and 5 μ M, roughly 400 fold better than compound **2** and 10 fold better than compound **1**. This observation suggests that the benzodiazepin portion of the molecule improves affinity for the enzyme active site, since the reactive motif of the molecule (the α -bromobenzylphosphonate) remains the same.

A series of molecules (Figure 2) are being prepared to be used in place of compound **12** in constructing a combinatorial library of inactivators. This synthetic method provides a rapid way of generating hundreds of molecules in a short period of time. These compounds will then be tested for activity against Yop51.

Scheme 2. Synthesis of compound **12**



Scheme 3. Solution synthesis of compound **3**

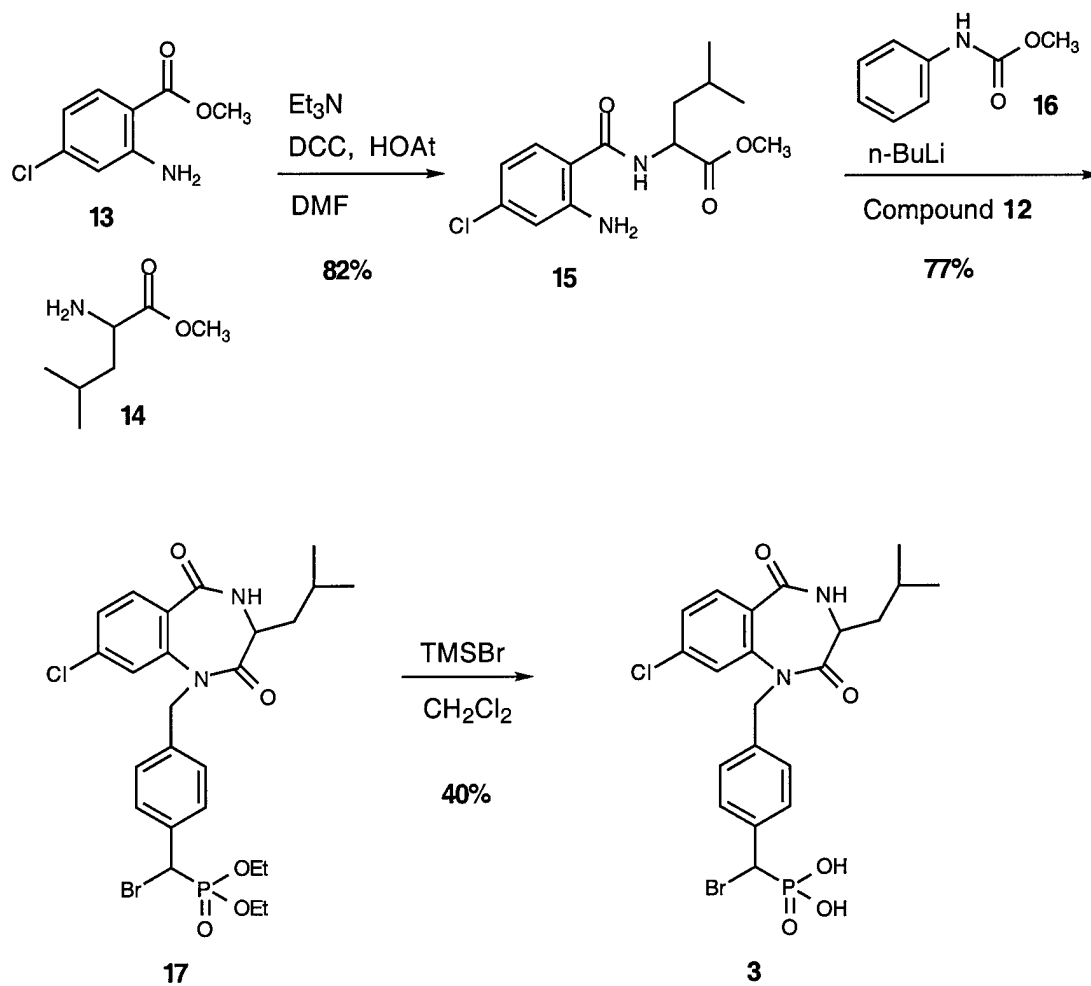
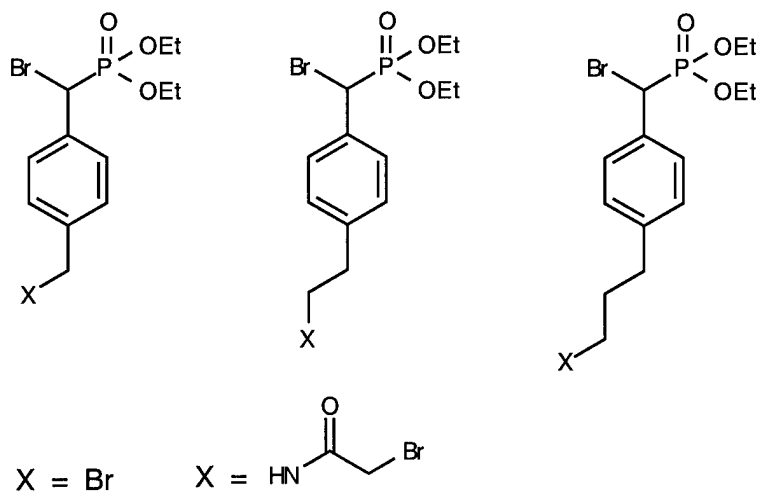


Figure 2. Analogs of compound **12**



Conclusions

Our study of inactivators of Yop51 has a number of important implications. (1) Non-peptidic inactivators of Yop51 may be useful for *in vitro* studies to delineate the role of PTPases in breast tumor development. (2) The enzyme appears to bind well to molecules which have an extended planar geometry. Compound **3** is the best novel inactivator for PTPases that has been synthesized to date.

The preparations and studies with the compounds described in this report are ongoing and may yield compounds suitable for both *in vitro* and *in vivo* studies on the efficacy of these inactivators in treatment of breast cancer.

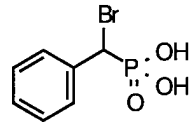
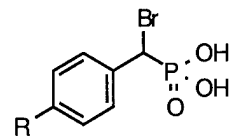
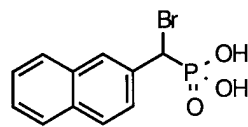
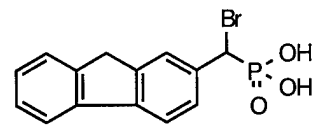
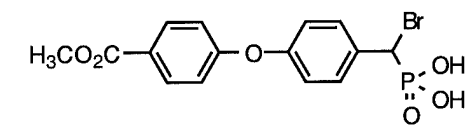
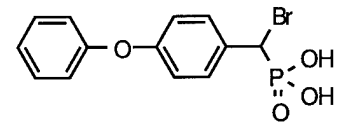
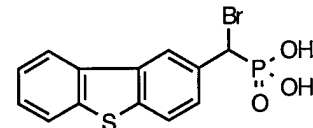
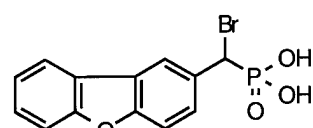
References

1. Glenny, J.R., Jr. *Biochem. Biophys. Acta* **113**, 1134 (1992); Fischer, E.H.; Charbonneau, D.R.; Tonks, N.K. *Science* **253**, 401 (1991).
2. Cyert, M.S.; Thorner, J. *Cell* **57**, 891 (1989); Banville, D.; Ahmad, S.; Stocco, R.; Shen, S. *J. Biol. Chem.* **269**, 22320 (1994).
3. Alexander, D.R. *New Biol.* **2**, 1049 (1990).
4. Zheng, X.M.; Wang, Y.; Pallen, C.J. *Nature* **359**, 336 (1992).
5. Verschuur, H.P.; Rijksen, G.; van Oirschot, B.A.; Schipper-Kester, G.P.M.; Slootweg, P.J.; Staal, G.E.J.; Hordijk, G.K. *Eur. Arch. Otorhinolaryngol.* **251**, 2 (1994); Wiener, J.R.; Hurteau, J.A.; Kearns, B.M.; Whitaker, R.S.; Conaway, M.R.; Berchuck, A.; Bast, R.C., Jr. *Am. J. Obstet. Gynecol.* **170**, 1177 (1994); Kidd, K.R.; Kerns, B.M.; Dodge, R.K.; Wiener, J.R. *J. Histochem. Cytochem.* **40**, 729 (1992).
6. Charbonneau, H.; Tonks, N.K.; Walsh, C.A.; Fischer, E.H. *Proc. Natl. Acad. Sci. USA* **85**, 7182 (1988).
7. Stukey, J.A.; Schuber, H.L.; Fauman, E.B.; Zhang, Z.; Dixon, J.E.; Saper, M.A. *Nature* **370**, 571 (1994).
8. Thomas, M.L. *Ann. Rev. Immunol.* **7**, 339 (1989).
9. Cho, H.; Krishnaraj, R.; Kitas, E.; Bannwarth, W.; Walsh, C.T.; Anderson, K.S. *J. Am. Chem. Soc.* **114**, 7296 (1992).
10. Bilwes, A.M.; den Hertog, J.; Hunter, T.; Noel, J.P. *Nature* **382**, 555 (1996); Barford, D.; Flint, A.J.; Tonks, N.K. *Science* **263**, 1397 (1994).
11. Zhang, Z-Y.; Dixon, J.E. *Biochemistry* **32**, 9340 (1993); Zhang, Z-Y.; Clemens, J.C.; Schubert, H.L.; Stuckey, J.A.; Fischer, M.W.F.; Hume, D.M.; Saper, M.A.; Dixon, J.E. *J. Biol. Chem.* **267**, 23759 (1992); Guan, K.; Dixon, J.E. *Science* **249**, 553 (1990).
12. Taylor, W.P.; Zhang, Z-Y.; Widlanski, T.S. *Bioor. Med. Chem. Lett.* **4**, 1515 (1996).

13. Wo, Y-Y.P.; Zhou, M-M.; Stevis, P.; Davis, J.P.; Zhang, Z-Y.; Van Etten, R.L. *Biochemistry* **31**, 1712 (1992).
14. Boojamra, C.G.; Burrow, K.M.; Thompson, L.A.; Ellman, J.A. *J. Org. Chem.* **62**, 1240 (1997).
15. Bunin, B.A.; Plunkett, M.J.; Ellman, J.A. *Methods Enzymol.* **267**, 448 (1996); DeWitt, S.H.; Kiely, J.S.; Stankovic, C.J.; Schroeder, M.C.; Cody, D.M.R.; Pavia, M.R. *Proc. Natl. Acad. Sci. USA* **90**, 6909 (1993).

APPENDIX 1

IC₅₀ TABLE

		IC ₅₀ Values (μM)
		1929
	R = NO ₂	164
	R = CN	166
	R = Br	800
	R = Cl	2098
	R = OCH ₃	2382
		2129
		116
		106
		310
		303
		39

APPENDIX 2

Yop51 Inactivation

